FORM PTO-1390 (REV 11-98) — U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 2799/64201

U.S. APPLICATION NO (If known, see 37 CFR 1.5): 09/762916

INTERNATIONAL PUBLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/NL99/00518	16 August 1999	NL 1009862 14 August 1998 NL 1010670 27 November 1998

TITLE OF INVENTION: Method for detecting a DNA sequence, a DNA sequence, a method of making a DNA construct and the use thereof

APPLICANT(S) FOR DO/EO/US: Otte, Arie Pieter

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371
3. [X] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [X] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [ ] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [ ] Other items or information:

17. [X] The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$970.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$840.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$760.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$670.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$96.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 860.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ] 20 [X] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	24 - 20 =	4	X **$18.00**	$ 72.00	
Independent claims	2 - 3 =	0	X **$78.00**	$	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$260.00**	$ 270.00	
TOTAL OF ABOVE CALCULATIONS =				$	
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also by filed (Note 37 CFR 1.9, 1.27, 1.28).				$	
SUBTOTAL =				$	
Processing fee of **$130.00** for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property				$	
TOTAL FEES ENCLOSED =				$ 1332.00	
				Amount to be: refunded	$
				charged	$

a. [X] A check in the amount of $ 1332.00 to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 03-3125. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO

Robert D. Katz
Cooper & Dunham LLP
1185 Avenue of the Americas
New York, New York 10036
(212) 278-0400

Robert D Katz
SIGNATURE

Robert D. Katz
NAME

30,141
REGISTRATION NUMBER

92750/58

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Stichting voor de Technische Wetenschappen
Serial No.	:	09/762,916
Int. Fil. Date	:	August 16, 1999
For	:	METHOD OF DETECTING A DNA SEQUENCE, A DNA SEQUENCE, A METHOD OF MAKING A DNA CONSTRUCT AND THE USE THEREOF

AMENDMENT IN RESPONSE TO APRIL 30, 2001 NOTIFICATION TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES

"Express Mail" mailing label No EL613247809US
Date of Deposit. June 29, 2001
I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1 10 on the date indicated above and is addressed to the Commissioner for Patents, Washington, D.C. 20231
Name: Lisa M. Melanson
Signature Lisa Melanson

Commissioner for Patents
Washington, D.C. 20231

Sir:

This Amendment is submitted in response to the Notification to Comply With Requirements for Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures that was issued on April 30, 2001 in connection with the above-identified application (attached hereto as Exhibit D). A response to the Notification to Comply is due on June 30, 2001. Accordingly, this Amendment is being timely filed.

Please amend the subject application as follows:

In the Specification:

Please replace the paragraph at page 7, line 11 with the following:

A vector coding for EBNA-1 (a nuclear antigen) is the hygromycin resistance gene comprising pREP4 vector (Invitrogen Corporation, Carlsbad, USA). The EBNA-1 sequence is present to ensure that the vector does not (stably) integrate in the genome, but replicates episomally. The promoter (Prsv) of this vector has been removed by digestion with the restriction enzyme SalI and replaced by a synthesized

sequence having four binding sites for LexA from *E. coli.* This sequence is from 5' - 3' : GTCGACTGCTGTATATAAAACCAGTGGTTATATGTACAGTACTTGTACTGTA CATATAACCACTGGTTTTATATACAGCAAGCTTGGATCCGTCGAC (SEQ ID NO:1). The 5' side of this sequence comprises a SalI site, the 3' side a HindIII-BamHI-SalI site (all shown in bold type). Downstream from the LexA binding sites in the HindIII and BamHI sites, the human heat shock factor-inducible promoter (0.29 kbp HindIII/NcoI fragment) and the luciferase reporter gene inclusive of SV40 polyadenylation signal (1.9 kbp NcoI/BamHI fragment) are cloned in a three-way ligation. The human heat shock factor-inducible promoter (hsp7O; accession numbers M59828 and M34267; nucleotides 52 to 244) can be obtained by means of PCR amplification on human genomic DNA (Cat. No. 6550-1; Clontech, Palo Alto, USA). As PCR primers, forward primer 5' - 3' : AAGCTTGGGAGTCGAAACTTCTGGAATATTCCCGAACTTTCAGCCGACG ACTTATAAAACGCCAGGGGCAAGC (SEQ ID NO:2) may be considered; and as reverse primer 5' - 3' : CCATGGTTTAGCTTCCTTAGCTCCTGAAAATCTCGCCAAGCTCCCGG GGTCCGCGAGAAGAGCTCGGTCCTTCCGG (SEQ ID NO:3). The forward primer comprises a HindIII site, the reverse primer comprises a NcoI site (given in bold print). The luciferase reporter gene inclusive of SV40 polyadenylation signals were obtained through NcoI/BamHI digestion of the pGL3 control vector (Cat. no E1741; Promega, Madison, USA). In the thus obtained vector, in the HindIII site between the LexA binding sites and the heat shock promoter, either a 2.1 kbp HindIII fragment of phage lambda is cloned (Pharmacia Biotech, Uppsala, Sweden), or a 1.7 kbp scs HindIII fragment. The 1.7 kbp scs DNA fragment is isolated from genomic *Drosophila* DNA (Cat. #6940-1, Clontech, Palo Alto, USA) with the aid of PCR primers (Forward primer 5' - 3' : GATCAAGCTTATGATCTGCGTATGATACCAAATTTCTG (SEQ ID NO:4); Reverse primer 5' - 3' : GACAAGCTTACATTGCTGGGCGAGCTGCGCCAATCG (SEQ ID NO:5)). At the ends of these primers HindIII restriction enzyme sites were located. The vector with the Lambda fragment (control) is indicated as reporter construct a, the vector with the scs fragment as reporter construct b. Restriction enzyme digestions,

PCR amplifications and clonings are performed by standard procedures as described in Sambrook et al., Molecular Cloning; a laboratory manual, second edition.

Please attach at the end of the application pages 1-3 of the Sequence Listing (attached hereto as Exhibit E).

REMARKS

By this Amendment, Applicant has amended the specification to refer to sequence identifiers, as required by the Sequence Rules, and to add the Sequence Listing. The amendments to the specification are supported by the application as originally filed. Accordingly, entry of the amendments to the specification is respectfully requested.

Compliance with Sequence Rules

The April 30, 2001 Notification to Comply With Requirements for Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures (Exhibit D) indicated that the application did not comply with the Sequence Rules. In response thereto, Applicant attaches herewith Exhibit F, consisting of pages 1-3 of the Sequence Listing. Also enclosed is a computer-readable form containing the Sequence Listing (Exhibit G). Additionally, the specification has been amended to contain the correct sequence identifiers, as required by the Sequence Rules.

The undersigned attorney hereby certifies that the information recorded in computer-readable form is identical to the written Sequence Listing, is supported by the application as filed, and does not introduce new matter into the application as filed. In view of the above-noted amendments and these remarks, applicant respectfully submits that he has complied with the Sequence Rules. Accordingly, entry of the Sequence Listing is respectfully requested.

No fee is deemed necessary in connection with the filing of this Amendment. If any fee is required to preserve the pendency of the application, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 01-1785.

Respectfully submitted,

AMSTER, ROTHSTEIN & EBENSTEIN
Attorneys for Applicant
90 Park Avenue
New York, New York 10016
(212) 697-5995

Dated: New York, New York
June 29, 2001

By: _____
Craig J. Arnold
Registration No. 34,287

SCHEDULE A

REDLINED VERSION

In the Specification:

Please replace the paragraph at page 7, line 11 as follows:

A vector coding for EBNA-1 (a nuclear antigen) is the hygromycin resistance gene comprising pREP4 vector (Invitrogen Corporation, Carlsbad, USA). The ~~De~~ EBNA-1 sequence is present to ensure that the vector does not (stably) integrate in the genome, but replicates episomally. The promoter ~~promotor~~ (Prsv) of this vector has been removed by digestion with the restriction enzyme SalI and replaced by a synthesized sequence having four binding sites for LexA from *E. coli.* This sequence is from 5' - 3' : GTCGACTGCTGTATATAAAACCAGTGGTTATATGTAC AGTACTTGTACTGTACATATAACCACTGGTTTTATATACAGCAAGCTTGGATCCGT CGAC (SEQ ID NO:1). The 5' side of this sequence comprises a SalI site, the 3' side a HindIII-BamHI-SalI site (all shown in bold type). Downstream from the LexA binding sites in the HindIII and BamHI sites, the human heat shock factor-inducible promoter ~~promotor~~ (0.29 kbp HindIII/NcoI fragment) and the luciferase reporter gene inclusive of SV40 polyadenylation signal (1.9 kbp NcoI/BamHI fragment) are cloned in a three-way ligation. The human heat shock factor-inducible promoter ~~promotor~~ (hsp7O; accession numbers M59828 and M34267; nucleotides 52 to 244) can be obtained by means of PCR amplification on human genomic DNA (Cat. No. 6550-1; Clontech, Palo Alto, USA). As PCR primers, forward primer 5' - 3' : AAGCTTGGGAG TCGAAACTTCTGGAATATTCCCGAACTTTCAGCCGACGACTTATAAAACGCCA GGGGCAAGC (SEQ ID NO:2) may be considered; and as reverse primer 5' - 3' : CCATGGTTTAGCTTCCTTAGCTCCTGAAAATCTCGCCAAGCTCCCGGGGTCCGCGAG AAGAGCTCGGTCCTTCCGG (SEQ ID NO:3). The forward primer comprises a HindIII site, the reverse primer comprises a NcoI site (given in bold print). The luciferase reporter gene inclusive of SV40 polyadenylation signals were obtained through

NcoI/BamHI digestion of the pGL3 control vector (Cat. no E1741; Promega, Madison, USA). In the thus obtained vector, in the HindIII site between the LexA binding sites and the heat shock promoter ~~promotor~~, either a 2.1 kbp HindIII fragment of phage lambda is cloned (Pharmacia Biotech, Uppsala, Sweden), or a 1.7 kbp scs HindIII fragment. The 1.7 kbp scs DNA fragment is isolated from genomic *Drosophila* DNA (Cat. #6940-1, Clontech, Palo Alto, USA) with the aid of PCR primers (Forward primer 5' - 3' : GATCAAGCTTATGATCTGCGTATGATACCAAATTTCTG (SEQ ID NO:4); Reverse primer 5' - 3' : GACAAGCTTACATTGCTGGGCGAGCTGCGCCAATCG (SEQ ID NO:5)). At the ends of these primers HindIII restriction enzyme sites were located. The vector with the Lambda fragment (control) is indicated as reporter construct a, the vector with the scs fragment as reporter construct b. Restriction enzyme digestions, PCR amplifications and clonings are performed by standard procedures as described in Sambrook et al., Molecular Cloning; a laboratory manual, second edition.

Dkt. 2799/64201

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Arie Pieter Otte

Application No.: 09/762,916

Filing Date: February 9, 2001

International Application No.: PCT/NL99/00518

International Filing Date: 16 August 1999

Title: Method of Detecting a DNA Sequence, a DNA Sequence, a Method of Making a DNA Construct and the Use Thereof

Sir:

PRELIMINARY AMENDMENT

Please cancel claims 1 - 24 without prejudice of disclaimer and substitute following new claims prior to examination:

IN THE CLAIMS:

25. (New) A method of detecting, and optionally selecting, a DNA sequence, wherein the DNA sequence to be detected possesses a stable expression-modulating quality, which method comprises the steps of :

1) cloning in a vector of DNA fragments between i) a DNA sequence involved in the induction of gene-transcription repressing chromatin, and ii) a reporter gene comprising a promotor, resulting in a variety of a fragment-comprising vectors;

2) introducing the vectors into a transcription system; and

3) subjecting the host cells to a selection step in order to identify the DNA sequence with a stable expression modulating quality.

26. (New) A method according to claim 25, wherein the DNA sequence comprises an expression-enhancing quality.

27. (New) A method according to claim 26, wherein the transcription system comprises host cells.

28. (New) A method according to claim 25, wherein the cloned DNA fragments have a size of 5,000 base pairs.

29. (New) A method according to claim 25, wherein the distance between the DNA sequence involved in gene repressing chromatin and the reporter gene is fewer than 5,000 base pairs.

30. (New) A method according to claim 25, wherein the promoter may be active in the transcription system but wherein induction of gene-repressing chromatin in the vectors results in the repression of the transcription of the reporter gene.

31. (New) A method according to claim 25, wherein the selection in step 3) occurs by using a reporter gene which provides resistance to a growth inhibitor.

32. (New) A method according to claim 31, wherein the host cells are cultivated in the presence of the growth inhibitor.

33. (New) A method according to claim 32, wherein the growth inhibitor is present in a concentration sufficiently high to kill host cells in which the gene providing resistance to the growth inhibitor is not active.

34. (New) A method according to claim 33, wherein an antibiotic is used as the growth inhibitor and the reporter gene provides resistance to the antibiotic.

35. (New) A method according to claim 34, wherein the reporter gene codes for Green Fluorescent Protein.

36. (New) A method according to claim 35, wherein the reporter gene is luciferase.

37. (New) A method according to claim 36, wherein the fluorescent host cells are separated from non-fluorescent host cells by means of a Fluorescence-Activated Cell Sorter (FACS).

38. (New) A method according to claim 29, wherein the cloned DNA fragments have a size of substantially between 2,000-3,000 base pairs.

39. (New) A method according to claim 25, wherein the DNA sequence involved with the transcription induction of gene-repressing chromatin is a DNA sequence that is recognized by a heterochromatin-binding protein comprising HP1, which HP1-comprising complex is present in the transcription system and/or the host cell.

40. (New) A method according to claim 25, wherein the DNA sequence involved with the transcription induction of gene-repressing chromatin is a DNA sequence that is recognized by a complex comprising a Polycomb-group (Pc-G) protein, and the Polycomb-group protein-comprising complex is present in the transcription system and/or in the host cell.

41. (New) A method according to claim 25, wherein the DNA sequence involved with the transcription induction of gene-repressing chromatin is a DNA sequence that is recognized by a complex possessing a histone deacetylase activity, and the histone deacetylase activity-possessing complex is present in the transcription system and/or in the host cell.

42. (New) A method according to claim 25, wherein the DNA sequence involved in the transcription induction of gene-repressing chromatin is a DNA sequence that is recognized by a protein complex comprising MeCP2 (methyl-CpG-binding protein 2), and the MeCp2-comprising complex is present in the transcription system and/or in the host cell.

43. (New) A method according to claim 25, wherein the DNA sequence involved with the transcription inducing of gene-repressing chromatin is a DNA sequence that is selectively recognized by at least one DNA-binding protein and the organism also expresses a protein complex comprising i) a first part selectively binding the DNA sequence, and ii) a second part inducing the formation of chromatin in which the transcription is repressed.

44. (New) A method according to claim 43, wherein the protein complex comprises a fusion protein.

45. (New) A method according to claim 44, wherein first part is a part binding the DNA binding site of LexA-DNA or GAL4-DNA.

46. (New) A method according to claim 25, wherein the organism in step 1) is selected from the group comprising a plant and a vertebrate.

47. (New) A method according to claim 46, wherein the vertebrate is a mammal.

48. (New) A method according to claim 25, wherein the vector is an episomally replicating vector.

49. (New) A method according to claim 48, wherein the vector comprises a replication origin from the Epstein-Barr virus (EBV), OriP, and a nuclear antigen (EBNA1).

50. (New) A DNA sequence selected from i) a DNA sequence isolated from a plant or vertebrate, or derivatives thereof, and ii) a synthetic DNA sequence or one constructed by means of genetic engineering, which DNA sequence is a repression-inhibiting sequence which, by the method according to the present invention can be detected, selected and optionally cloned.

51. (New) A DNA sequence selected from i) a DNA sequence isolated from a plant or vertebrate, or derivatives thereof, and ii) a synthetic DNA sequence is detected, selected and optionally cloned by the method set forth in claim 25.

52. (New) A method of making a DNA construct comprising a gene that is to be expressed stably, wherein a stable expression-promoting DNA sequence is integrated in accordance with claim 51.

53. (New) A method according to claim 52, wherein the DNA sequence is integrated in fewer than 2,000 bp of the gene.

54. (New) A method according to claim 52, wherein the stable expression-modulating DNA sequence will be integrated both upstream and downstream from the gene.

55. (New) A method according to claim 54 wherein the DNA sequence comprises an expression enhancing DNA sequence.

REMARKS

This preliminary amendment cancels claims 1-24 without prejudice and presents new claims 25-55. These claims contain no new matter. A check in the amount of $144.00 is enclosed herewith to cover the fees for the additional claims requested. Entry of the amendment is respectfully requested.

Any deficiency in payment or supplemental fees should be charged to our deposit account No. 03-3125.

Respectfully submitted,

Dated: March 27, 2001

By: Robert D Katz

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Dkt. 2799/64201

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Arie Pieter Otte

International Application No.: PCT/NL99/00518

International Filing Date: 16 August 1999

Title of Invention: Method of Detecting a DNA Sequence, a DNA Sequence, a Method of Making a DNA Construct and the Use Thereof

PRELIMINARY AMENDMENT

Please amend the application as follows before examination:

IN THE CLAIMS:

In claim 8, delete "any of the preceding claims", and insert --claim 4 -- therefor.

In claim 9, delete "any of the preceding claims"' and insert -- claim 8 -- therefor.

In claim 10, delete "any of the claims 1 to 8", and insert --claim 8-- therefor.

In claim 11, delete "any of the claims 1 to 8", and insert --claim 8-- therefor.

In claim 12, delete "any of the claims 1 to 8" and insert --claim 8-- therefor.

In claim 13, delete "any of the preceding claims and insert -- claim 8 -- therefor.

In claim 18, delete "any of the preceding claims and insert -- claim 8 -- therefor.

In claim 19, delete "any of claims 1 to 19", and insert --claim 18-- therefor.

In claim 21, delete "any of claims 1 to 19", and insert --claim 18-- therefor.

In claim 22, delete "20 or".

In claim 24, delete "or 23"

REMARKS

This preliminary amendment deletes improper multiple dependency, and adds no new matter. Its entry is respectfully requested.

Any deficiency in payment or supplemental fees should be charged to our deposit account No. 03-3125.

Respectfully submitted,

Dated: 2-09-01

By: _____

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Docket No. 2799/64201

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

(U.S. DESIGNATED OFFICE)

APPLICATION FOR

UNITED STATES LETTERS PATENT

BASED UPON:
INTERNATIONAL APPL. NO. PCT/NL99/00518
UNDER PATENT COOPERATION TREATY

INVENTOR: Otte, Arie Pieter

TITLE: Method for detecting a DNA sequence, a DNA sequence, a method of making a DNA construct and the use thereof

ATTORNEY: Robert D. Katz
Reg. No. 30,141
COOPER & DUNHAM LLP
1185 Avenue of the Americas
New York, New York 10036
(212) 278-0400
Attorney for Applicant

Method of detecting a DNA sequence, a DNA sequence, a method of making a DNA construct and the use thereof

The present invention relates to a method of detecting, and optionally selecting, a DNA sequence.

It is not easy to detect a specific DNA sequence of which the nucleotide sequence is not known. Despite the fact that genetic manipulation has been employed for decades, predictably bringing to expression a gene in a genetically modified plant, animal or other eukaryotic organism is a problem. Although many microbiological methods of production merely aim at the highest possible expression, in plants or animals the exact level of a gen's expression is for many applications of great importance. Too much expression as well as too little expression may lead to the desired result not being achieved. Also, experience has shown that after sexual reproduction the ability for expression in a subsequent generation is often lost again. It is also difficult to control the moment in time and the location of expression in the organism (tissue specificity).

It is the object of the invention to provide a method of the kind mentioned in the preamble, which makes it possible to select and, if desired, isolate a DNA sequence, whereby the above-mentioned problems can be avoided.

To this end the method according to the preamble is characterized in that the DNA sequence to be detected possesses a stable expression-enhancing quality, which method comprises the steps of

1) the cloning in a vector of DNA fragments having a size of <5000 base pairs between i) a DNA sequence involved in the induction of gene transcription-repressing chromatin, and ii) a reporter gene comprising a promotor, resulting in a variety of a fragment-comprising vectors, wherein the distance between the DNA sequence involved in the induction of the transcription of

gene-repressing chromatin and the reporter gene is fewer than 5000 base pairs;

2) introducing the vectors into host cells, in which host cells the promotor may be active but induction of the transcription of gene-repressing chromatin in the vectors results in the repression of the transcription of the reporter gene; and

3) subjecting the host cells to a selection in order to identify a host cell exhibiting reporter gene-activity.

This provides a reliable method of detecting DNA sequences having a stable expression-enhancing quality. If desired, this sequence may be isolated and inserted before another gene. As the DNA in step 1, for example, a restriction enzyme-cleaved DNA from a eukaryotic organism, in particular a plant or a vertebrate, is used wherein the size of the DNA fragments is below 5000 base pairs.

Clearly, when the occasion arises it will be possible to readily distinguish between on the one hand an expression-enhancing sequence ("enhancer"), which in extreme cases would be able to neutralize the transcription-repressing effect of chromatin, and on the other hand the stable expression-enhancing DNA fragment. In the first case the reporter gene in an organism is transformed with a vector comprising the promotor together with the reporter gene but without the transcription-repressing sequence is expressed at a higher level than in an organism transformed with a vector comprising a stable expression-enhancing DNA fragment together with the reporter gene and likewise, without the transcription-repressing sequence.

According to a first preferred embodiment, the selection in step 3) occurs by using a reporter gene which provides resistance to a growth inhibitor and the host cells are cultivated in the presence of the growth inhibitor.

This inhibits the growth of host cells which, without an active resistance gene, are not resistant to the growth inhibitor, and allows the selection of those host

cells which possess a stable expression-enhancing DNA sequence.

Preferably, the growth inhibitor is present in a concentration sufficiently high to kill host cells in which the gene providing resistance to the growth inhibitor is not active.

This ensures to a large extent that growing organisms will comprise a vector with the desired DNA sequence.

Very conveniently an antibiotic is used as the growth inhibitor and the reporter gene is a gene providing resistance to the antibiotic.

A great assortment of genes providing resistance to antibiotics is available in the field, making it simple to choose a gene suitable for the host cell. A gene is then chosen which provides resistance to a growth inhibitor to which the host cell is not already resistant of itself.

In accordance with a second embodiment the reporter gene codes for Green Fluorescent Protein.

By means of fluorescence measurement it is then possible to detect and isolate host cells with the desired DNA-comprising vector.

According to a preferred embodiment, fluorescent host cells are separated from non-fluorescent host cells by means of a Fluorescence-Activated Cell Sorter (FACS).

According to a third embodiment the reporter gene is luciferase. With the aid of luciferase it is possible to perform (semi)-quantitative measurement of the expression.

In step 1) it is preferred that the fragments have a size of substantially between 2000 - 3000 base pairs.

Fragments of such a size allow a more precise localization of the sequence to be detected without the number of host cells to be screened in step 3) becoming so large that this is going to form an unnecessary work load.

Conveniently, the DNA sequence involved with the transcription induction of gene-repressing chromatin is a DNA sequence that is recognized by a heterochromatin-binding protein comprising HP1 (heterochromatin-binding protein 1), which HP1-comprising complex is expressed in the

host cell. According to an alternative method, the DNA sequence is recognized by a complex comprising a Polycomb-group (Pc-G) protein, and the Polycomb-group protein-comprising complex is expressed in the host cell. According to yet another embodiment, the DNA sequence is recognized by a complex possessing a histone deacetylase activity, and the histone deacetylase activity-possessing complex is expressed in the host cell. Finally, according to a further embodiment, the DNA sequence involved in the induction of the transcription of gene-repressing chromatin is a DNA sequence recognized by a protein complex comprising MeCP2 (methyl-CpG-binding protein 2), and the MeCP2-comprising complex is expressed in the host cell.

In this manner four suitable complexes recognizing DNA sequences are provided, while it should be noted that in the event of the complex not being expressed in the host cell, this will not result in false positives and will merely limit the efficiency with which the wanted DNA sequences are detected.

Conveniently, the protein complex comprises a fusion protein, such as a protein complex wherein the first part is a part binding the DNA-binding site of LexA-DNA or GAL4-DNA.

Suitable DNA binding sites of this kind are known in the art and are obtained from bacteria or yeast.

The organism in step 1) is preferably chosen from the group comprising a plant and a vertebrate such as, more particularly, a mammal.

For these organisms applies that, partly due to the large amount of chromosomal DNA, it is practically impossible without the method of the present invention to find the DNA sequence to be detected, since indeed its base sequence is unknown.

According to a further preferred embodiment, the vector is an episomally replicating vector, such as suitably a vector comprising a replication origin from the Epstein-Barr virus (EBV), OriP, and a nuclear antigen (EBNA1).

Such vectors are easy to handle, can be genetically manipulated and are vectors which form a chromatin structure in which the expression is repressed.

The invention further relates to a DNA sequence selected from i) a DNA sequence isolated from a plant or vertebrate, or derivatives thereof, and ii) a synthetic DNA sequence or one constructed by means of genetic engineering, which DNA sequence is a repression-inhibiting sequence which, by the method according to the present invention can be detected, selected and optionally cloned.

More specifically, the invention further relates to a DNA sequence selected from i) a DNA sequence isolated from a plant or vertebrate, or derivatives thereof, and ii) a synthetic DNA sequence or one constructed by means of genetic engineering, which DNA sequence is detected, selected and optionally cloned by the method according to the present invention.

The DNA sequences according to the invention differ from the known DNA sequences in that they are not an enhancer or silencer.

Synthetic DNA sequences may be prepared in accordance with techniques generally known in the art. In particular, it is possible to prepare large numbers of different DNA sequences, and such sequences are commercially available (for example from: Pharmacia Biotech, Uppsala, Sweden). However, such synthetic DNA sequences have to be suitable for cloning in a plasmid. This is generally known in the art and is done, for example, with linkers comprising a restriction cleavage site.

Clearly, the present invention also relates to a method of making a DNA construct comprising a gene that is to be expressed stably, wherein a stable expression-enhancing DNA sequence, selected with the aid of the method according to the invention is inserted at less than 2000 bp from the gene.

This is a more stable and predictable manner of expressing a gene.

Preferably the stable expression-enhancing DNA sequence will be inserted both upstream and downstream from the gene.

It is believed that this further increases the likelihood of a stable gene expression.

Finally, the invention relates to a use of the DNA construct according to the invention, wherein the DNA construct is a vector, for the transformation of an organism which suitably is an organism as defined above.

The present invention will now be further elucidated with reference to the following exemplary embodiments.

Example I

To illustrate the principle of the workings of the method according to the invention, scs is used, which is a DNA fragment from *Drosophila melanogaster* which is known to be a boundary element. As can be seen from the example below, scs can be used for blocking the following repressors: HP1, Polycomb-group proteins and MeCP2. In the same manner, DNA fragments from phage lambda have been tested as negative control. Scs (special chromatin structure) was originally isolated as a DNA sequence flanking the heat shock locus (*hsp*70) in *Drosophila* (Kellum, R. and P. Schedl. 1991. Cell 64: 941-950). They have found that when scs is placed around a reporter gene and is reintroduced in *Drosophila*, the expression of a reporter gene is less variable. They neither reported nor suggested that scs may be used to prevent repression by other repressors, in particular the above-mentioned repressors. Also, Kellum et al. neither reported nor suggested that scs might be used in systems other than *Drosophila* for rendering transgene expression less variable.

For testing the repression-eliminating property of a DNA sequence, two types of vectors are constructed.

The first type of vector comprises in 5'-3' sequence: four LexA binding sites, the scs sequence to be tested, the human heat shock factor-inducible promotor, and the luciferase gene as reporter gene. As a control a

similar vector is made which instead of the known scs sequence comprises a random fragment (from phage lambda) of a comparable length (both described in point 1 below).

To accomplish repression of the reporter gene in the transformed cell, the second type of vector comprises a gene coding for a fusion protein of LexA and the above-mentioned repressors. A vector of this second type comprises the gene coding for LexA only, or a vector comprises the gene coding for LexA-HP1, etc. (described in point 2 below).

1 A vector coding for EBNA-1 (a nuclear antigen) is the hygromycin resistance gene comprising pREP4 vector (Invitrogen Corporation, Carlsbad, USA). De EBNA-1 sequence is present to ensure that the vector does not (stably) integrate in the genome, but replicates episomally. The promotor (Prsv) of this vector has been removed by digestion with the restriction enzyme SalI and replaced by a synthesized sequence having four binding sites for LexA from *E. coli*. This sequence is from 5'-3': **GTCGAC**TGCTGTATATAAAACCAGTGGTTATATGTACAGTACTT GTACTGTACATATAACCACTGGTTTTATATACAG-**CAAGCTTGGATCCGTCGAC**. The 5' side of this sequence comprises a SalI site, the 3' side a HindIII-BamHI-SalI site (all shown in bold type). Downstream from the LexA binding sites in the HindIII and BamHI sites, the human heat shock factor-inducible promotor (0.29 kbp HindIII/NcoI fragment) and the luciferase reporter gene inclusive of SV40 polyadenylation signal (1.9 kbp NcoI/BamHI fragment) are cloned in a three-way ligation. The human heat shock factor-inducible promotor (hsp70; accession numbers M59828 and M34267; nucleotides 52 to 244) can be obtained by means of PCR amplification on human genomic DNA (Cat. No. 6550-1; Clontech, Palo Alto, USA). As PCR primers, forward primer 5'-3': **AAGCTTGGGAGTCGAAACTTCTGGAATATTCCCGAACTTTCAGCCGACGA-**CTTATAAAACGCCAGGGGCAAGC may be considered; and as reverse primer 5'-3': **CCATGG**TTTAGCTTCCTTAGCTCCTGAA-

AATCTCGCCAAGCTCCCGGGGTCCGCGAGAAGAGCTCGGTCCTTCCGG. The forward primer comprises a HindIII site, the reverse primer comprises a NcoI site (given in bold print). The luciferase reporter gene inclusive of SV40 polyadenylation signals were obtained through NcoI/BamHI digestion of the pGL3 control vector (Cat. no E1741; Promega, Madison, USA). In the thus obtained vector, in the HindIII site between the LexA binding sites and the heat shock promotor, either a 2.1 kbp HindIII fragment of phage lambda is cloned (Pharmacia Biotech, Uppsala, Sweden), or a 1.7 kbp scs HindIII fragment. The 1.7 kbp scs DNA fragment is isolated from genomic *Drosophila* DNA (Cat.#6940-1, Clontech, Palo Alto, USA) with the aid of PCR primers (Forward primer 5'-3': GATCAAGC-TTATGATCTGCGTATGATACCAAATTTCTG; Reverse primer 5'-3': GACAAGCTTACATTGCTGGGCGAGCTGCGCCAATCG). At the ends of these primers HindIII restriction enzyme sites were located. The vector with the Lambda fragment (control) is indicated as reporter construct a, the vector with the scs fragment as reporter construct b. Restriction enzyme digestions, PCR amplifications and clonings are performed by standard procedures as described in Sambrook et al., Molecular Cloning; a laboratory manual, second edition.

2 The DNA-binding domain of the LexA protein (aa 1-202) (Cat.#6183-1, Clontech, Palo Alto, USA) is cloned in the HindIII site of the neomycin resistance gene-comprising pREP9 (Invitrogen Corporation, Carlsbad, USA) vector. Downstream and in frame with the LexA gene, one gene coding for a repressor is cloned per vector. The repressors used are: the 1674 bp-long coding part of the humane Polycomb-group gene HPC2 (accession number Genbank: AAB80718), the 1131 bp-long coding part of the humane Polycomb-group gene RING1 (accession number Genbank: Z14000), the 4098 bp-long coding part of

the *Drosophila* Polycomb-group gene Su(z)2 (accession number Genbank: CAA41965), the 558 bp coding part of *M31 (mHP1)* (accession number Genbank: P23197), or the 1478 bp coding part of *MeCP2* (accession number Genbank: A41907). These constructs code for LexA-HPC2, LexA-RING1, LexA-Su(z)2, LexA-mHP1 and LexA-MeCP2 fusion proteins, or **LexA repressors**. These bind to the LexA binding sites (see point 1).

3 The reporter vectors a and b and the LexA repressor-coding vectors are expressed in humane U-2 OS (osteosarcoma) cells obtained from the ATTC (accession number HTB-96). Transfection of the cells with the DNA constructs is performed using the calcium phosphate method in accordance with the instructions of the manufacturer of the transfection kit (Cat. No. 18306-019, Gibco BRL, Gaithersburg, USA). The osteosarcoma cells grow in the presence of 100 µg/ml neomycin (G418: Cat. No. 1464981; Boehringer/Roche, Switzerland) and 50 µg/ml hygromicin B (Cat. No 843555; Boehringer/Roche, Switzerland). Three days after transfection a heat shock is given (43°C for 1 hour, followed by a 6-hour recovery period at 37°C). This treatment activates the luciferase gene and causes the production of the luciferase reporter protein. The enzymatic activity of this luciferase protein is a measure of the transcription induction that has been induced. Cells are purified and the luciferase enzyme activity is measured, all in compliance with the manufacturer's instructions for the standard luciferase reporter gene assay kit (Cat. No. 1814036; Boehringer/Roche, Switzerland).

Result

4 In cells in which the reporter construct a (with the Lambda fragment) is expressed, but no LexA

repressors, the luciferase gene is expressed after heat shock. This is the 100% value.

5 In cells in which the reporter construct b (with the scs fragment) is expressed, but no LexA repressors, the luciferase gene is expressed after heat shock up to a value of 100%. Since this value does not exceed the 100% it shows, as explained earlier, that it is not an expression-increasing sequence.

6 In cells in which the reporter construct a (with the Lambda fragment) is expressed, and also LexA repressors are expressed, the expression of the luciferase gene after heat shock is repressed to an average of 20%.

7 In cells in which the reporter construct b (with scs fragment) is expressed, and at the same time LexA repressors, the expression of the luciferase gene after heat shock reaches a value of 100%. This shows that the induction of the repressor activity can be repressed with scs.

Example II

Instead of luciferase as reporter gene, it is according to the present invention also possible to use another reporter gene. It is also possible to use other promotors.

8 In the reporter constructs a and b the luciferase reporter gene has been replaced by the Zeocin resistance gene. The heat shock promotor has been replaced by the constitutive SV40 promotor (pSV40/ZEO; Cat. No. V502-20; Invitrogen, Carlsbad, USA). After transfection the U-2 OS cells grow in 250 µg/ml Zeocin (Cat. No. R250-01: Invitrogen, Carlsbad, USA) and 100 µg/ml neomycin (G418: Cat. No. 1464981; Boehringer/Roche, Switzerland).

9 Cells that have been transfected with the selection construct comprising a 2.1 kbp Lambda fragment and also with a construct that expresses a LexA repressor, die after 20-30 days. This shows that

the Lambda fragment is not able to overcome the repression of the gene with which antibiotics resistance is achieved.

10 Cells that are transfected with the selection construct comprising the scs fragment and also with a construct that expresses a LexA repressor, do not die but continue to grow. This also shows that with the boundary element scs the repression can be overcome and that the method according to the present invention can be employed using a variety of promotors and reporter genes.

Example III

The sequences found and selected by the method according to the invention can be used to combat repression in an organism other than that from which the sequence is derived.

11 Two new constructs, c and d, are made, so-called T-DNA constructs, which are suitable for the transformation of plants.

12 Construct c comprises a cassette with the NPTII (neomycin phosphotransferase II) gene for resistance selection with kanamycin and the GUS (β-glucuronidase) reporter gene. The NPTII gene is regulated by the constitutive nos promotor and the GUS reporter gene by the constitutive CaMV 35S promotor (Mlynarova, L. et al., 1995. The Plant Cell 7: 599-609).

13 Construct d is construct c in which an scs fragment is cloned immediately upstream from the GUS-CaMV/nos-NPTII cassette and an scs fragment immediately downstream from the cassette.

14 Agrobacterium tumefaciens is transformed with construct c or d. *Arabidopsis* plants are submerged in a suspension (culture) of *Agrobacterium tumefaciens* with construct c and in a suspension of *Agrobacterium tumefaciens* with construct d (Clough et al., 1998. The Plant J. 16: 735-743).

40 individual *Arabidopsis* plants with construct c or d are raised and the seeds of the plants collected. The seeds are sown onto a medium containing kanamycin (Cat. No. 106801; Boehringer/Roche, Swiss) and GUS reporter activity is measured in the leaves of the developed plants.

16 The GUS activity in plants with construct c is very variable (7 high; 6 intermediate; 11 low; 16 zero); the GUS activity in plants with construct d is systematically higher and the variability is reduced (26 high; 4 intermediate; 5 low; 5 zero).

17 This shows that a gene can be expressed more stably with a boundary element, even if this boundary element does not originate from the same organism.

CLAIMS

1. A method of detecting, and optionally selecting, a DNA sequence, **characterized** in that the DNA sequence to be detected possesses a stable expression-enhancing quality, which method comprises the steps of

1) the cloning in a vector of DNA fragments having a size of <5000 base pairs between i) a DNA sequence involved in the induction of gene-transcription repressing chromatin, and ii) a reporter gene comprising a promotor, resulting in a variety of a fragment-comprising vectors, wherein the distance between the DNA sequence involved in the induction of the transcription of gene-repressing chromatin and the reporter gene is fewer than 5000 base pairs;

2) introducing the vectors into host cells, in which host cells the promotor may be active but induction of the transcription of gene-repressing chromatin in the vectors results in the repression of the transcription of the reporter gene; and

3) subjecting the host cells to a selection in order to identify a host cell exhibiting reporter gene-activity.

2. A method according to claim 1, **characterized** in that the selection in step 3) occurs by using a reporter gene which provides resistance to a growth inhibitor and the host cells are cultivated in the presence of the growth inhibitor.

3. A method according to claim 2, **characterized** in that the growth inhibitor is present in a concentration sufficiently high to kill host cells in which the gene providing resistance to the growth inhibitor is not active.

4. A method according to claim 2 or 3, **characterized** in that an antibiotic is used as the growth inhibitor and the reporter gene is a gene providing resistance to the antibiotic.

5. A method according to claim 1, **characterized** in that the reporter gene codes for Green Fluorescent Protein.

6. A method according to claim 5, **characterized** in that fluorescent host cells are separated from non-fluorescent host cells by means of a Fluorescence-Activated Cell Sorter (FACS).

7. A method according to claim 1, **characterized** in that the reporter gene is luciferase.

8. A method according to any of the preceding claims, **characterized** in that the fragments have a size of substantially between 2000 - 3000 base pairs.

9. A method according to any of the preceding claims, **characterized** in that the DNA sequence involved with the transcription induction of gene-repressing chromatin is a DNA sequence that is recognized by a heterochromatin-binding protein comprising HP1 which HP1-comprising complex is expressed in the host cell.

10. A method according to any of the claims 1 to 8, **characterized** in that the DNA sequence involved with the transcription induction of gene-repressing chromatin is a DNA sequence that is recognized by a complex comprising a Polycomb-group (Pc-G) protein, and the Polycomb-group protein-comprising complex is expressed in the host cell.

11. A method according to any of the claims 1 to 8, **characterized** in that the DNA sequence involved with the transcription induction of gene-repressing chromatin is a DNA sequence that is recognized by a complex possessing a histone deacetylase activity, and the histone deacetylase activity-possessing complex is expressed in the host cell.

12. A method according to any of the claims 1 to 8, **characterized** in that the DNA sequence involved in the transcription induction of gene-repressing chromatin is a DNA sequence that is recognized by a protein complex comprising MeCP2 (methyl-CpG-binding protein 2), and the MeCP2-comprising complex is expressed in the host cell.

13. A method according to any of the preceding claims, **characterized** in that the DNA sequence involved with the transcription induction of gene-repressing

chromatin is a DNA sequence that is selectively recognized by at least one DNA-binding protein and the organism also expresses a protein complex comprising i) a first part selectively binding the DNA sequence, and ii) a second part inducing the formation of chromatin in which the transcription is repressed.

14. A method according to claim 13, **characterized** in that the protein complex comprises a fusion protein.

15. A method according to claim 14, **characterized** in that the first part is a part binding the DNA-binding site of LexA-DNA or GAL4-DNA.

16. A method according to any of the preceding claims, **characterized** in that the organism in step 1) is selected from the group comprising a plant and a vertebrate.

17. A method according to claim 16, **characterized** in that the vertebrate is a mammal.

18. A method according to any of the preceding claims, **characterized** in that the vector is an episomally replicating vector.

19. A method according to any of the preceding claims, **characterized** in that the vector comprises a replication origin from the Epstein-Barr virus (EBV), OriP, and a nuclear antigen (EBNA1).

20. A DNA sequence selected from i) a DNA sequence isolated from a plant or vertebrate, or derivatives thereof, and ii) a synthetic DNA sequence or one constructed by means of genetic engineering, which DNA sequence is a repression-inhibiting sequence which, by the method according to the present invention can be detected, selected and optionally cloned.

21. A DNA sequence selected from i) a DNA sequence isolated from a plant or vertebrate, or derivatives thereof, and ii) a synthetic DNA sequence or one constructed by means of genetic engineering, which DNA sequence is detected, selected and optionally cloned by means of the method according to any of the claims 1 to 19.

22. A method of making a DNA construct comprising a gene that is to be expressed stably, wherein a stable

expression-promoting DNA sequence is integrated in accordance with claim 20 or 21 in fewer than 2000 bp of the gene.

23. A method according to claim 22, characterized in that the stable expression-enhancing DNA sequence will be integrated both upstream and downstream from the gene.

24. A use of the DNA construct obtained in accordance with claim 22 or 23, wherein the DNA construct is a vector for the transformation of an organism.

M. H

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(57) Abstract

The invention relates to a method of detecting a DNA sequence which at least partially contributes to promote the stable expression of a gene. To this end the DNA fragment to be examined is cloned in a vector between i) a DNA sequence involved in the induction of gene transcription repressing chromatin, and ii) a reporter gene. The invention also relates to a DNA sequence to be detected by means of the invention, and the application of a stable expression–enhancing DNA sequence for the stable expression of a gene.

Declaration and Power of Attorney Patent Application (Design or Utility)

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I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: "Method of Detecting a DNA sequence, a DNA Sequence, a Method of Making a DNA Construct and the use Thereof."

the specification of which

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x was filed on February 9, 2001, as application serial no. 09/762,916 and or PCT International Application number PCT/NL99/00518 and was amended on (if applicable).

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Number 1009862	**Country** NL	**Day/Month/Year Filed** 14 August 1998
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